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<b>(21) International Application Number:</b> PCT/AU89/00038 <b>(22) International Filing Date:</b> 3 February 1989 (03.02.89)  <b>(31) Priority Application Number:</b> PI 6612 <b>(32) Priority Date:</b> 5 February 1988 (05.02.88) <b>(33) Priority Country:</b> AU  <b>(71) Applicant (for all designated States except US):</b> COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, 2601 ACT (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> BOTH, Gerald, Wayne [AU/AU]; 4 Leslie Street, North Ryde, NSW 2113 (AU). WHITFELD, Peter, Lawrence [AU/AU]; Flat 10, 29 Leichardt Street, Glebe, NSW 2037 (AU). STIRZAKER, Sally, Clare [AU/AU]; 4 Matherson Ave, Chatswood, NSW 2067 (AU).		<b>(74) Agent:</b> F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> GENE EXPRESSION SYSTEM (PARTICULARLY FOR ROTAVIRUS VP7 PROTEIN) INVOLVING A FOREIGN SIGNAL PEPTIDE AND OPTIONALLY A TRANSMEMBRANE ANCHOR SEQUENCE  <b>(57) Abstract</b>  <p>The present invention relates to novel genes for the expression of proteins. These genes enable the expression of proteins which are normally retained within a cell to either be exported from the cell or bound to the cell membrane of the cell. In addition, novel genes are provided for the expression of proteins, which are naturally exported from the cell, bound to the cell membrane. The novel genes of the present invention are particularly useful in the expression of antigens of rotavirus and particularly to rotavirus VP7 protein which is normally retained in the endoplasmic reticulum of the cell. By enabling the expression of proteins such as rotavirus VP7 protein on the cell membrane or in a form exported from the cell an advantageous method is provided for obtaining this antigen for use in vaccines.</p>		

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Gene expression system (particularly for rotavirus VP7 protein) involving a foreign signal peptide and optionally a transmembrane anchor sequence.

The present invention relates to novel genes for the expression of proteins and in particular novel genes expressing antigens of rotaviruses and more particularly to VP7 antigens having altered amino acid sequences.

Rotaviruses are a major cause of diarrheal disease. Worldwide they account for some 140 million cases of illness annually with an associated one million deaths (Robbins & Freeman Sci. Am. 259:126 1988). Approximately 50% of hospitalized cases of diarrheal illness for children in the 6-24 months age group in the U.S.A., Japan and Australia are rotavirus induced (Kapikian & Channock 1985) pp 863-906 in "Virology" B N Fields (Ed) Raven Press, New York.

Intensive research has been done on these viruses in the past seven years. The viral genome and proteins have been described and genetic studies have shown that the viral antigen, VP7, which induces the formation of neutralizing antibodies is coded for by gene segment 8 or 9, depending on the strain of rotavirus.

Since VP7 is the major viral protein against which neutralizing antibodies are directed, it is a prime candidate for the development of a rotavirus vaccine affording protection through a single viral protein.

The present inventors have investigated the virus on a molecular level with a view to developing a rotavirus vaccine based on recombinant DNA technology. It was earlier found by cloning and sequencing rotavirus dsRNA genome segments, that segment 9 is the one which codes for VP7 protein in the Simian rotavirus SA11. The equivalent genes describing the VP7 proteins for a human strain S2 and a bovine strain NCDV have also been cloned and sequenced (Both et al P.N.A.S. 80:3091-3095, 1983; Gunn et al, J. Virology 54:791-797, 1985).

Comparison of the VP7 proteins as deduced from the gene sequences reveals certain features which are conserved, notably two regions of hydrophobic amino acids

H1 and H2 near the amino terminus. These are involved in directing newly synthesized VP7 to its correct location in the cell for virus assembly. Virus particles which are partially assembled in the cell cytoplasm migrate to the membrane of the rough endoplasmic reticulum (ER). The immature particles then bud through the membrane becoming transiently enveloped as they do so. The VP7 protein may be acquired at this time or later when the enveloping membrane is lost. The VP7 protein is therefore unusual in that it is retained in the ER for virus assembly. Most other viral glycoproteins are transported to the cell surface membrane or directed to other organelles in the cell. In fact there is no evidence for transport of VP7 beyond the ER of the infected cell.

It was previously found that by modifying the amino acid sequence of VP7, it was possible to produce a protein which was now transported out of the ER and secreted into the extracellular medium (Poruchynsky et al., J. Cell Biol. 101:2199-2209, 1985). However, the basis for this secretion was not understood. Recently, the present inventors elucidated the location of the signal peptide sequences in VP7 which are responsible for directing the protein to the ER (Whitfield et al. Molec. and Cell. Biol. 7:2491-2497, 1987). The site at which the signal peptide is cleaved from the precursor protein to yield mature VP7 was also determined using genetic engineering and protein sequencing techniques (Stirzaker et al. J. Cell Biol. 105: 2897-2903, 1987). It is now clear that glutamine residue 51 (numbered according to the codons in the open reading frame of the VP7 gene) is the N-terminal residue of mature VP7. It is predicted (Von Heijne Nuc. Ac. Res 14: 4683-4699 1988) that this cleavage site is conserved in all serotypes of VP7 whose structure has been determined (Gorziglia, et al. J. Gen. Virol. 67:2445-2454, 1986).

With the elucidation of this cleavage site, the

earlier data of Poruchynsky et al (1985) were reassessed. Specifically, the effect of two internal deletions on the targeting of VP7 to the ER was compared. One mutation, which deleted amino acids 51-61 inclusive had no effect on the retention of VP7 in the ER. However, another which deleted residues 47-61 inclusive resulted in the rapid secretion of VP7 into the medium. With knowledge of the cleavage site it became clear that the former mutation left intact the H2 hydrophobic domain (signal peptide) which directs VP7 across the ER membrane (Whitfield et al, Molec. and Cell. Biol. 7:2491-2903, 1987). The 47-61 mutation, however, truncated the H2 region. These data suggested the unprecedented possibility that the H2 signal peptide was involved both in directing VP7 to the ER and retaining it there. The present inventors therefore examined this.

The H2VP7 signal peptide (comprising residues 30-50 of the open reading frame) (Fig. 1) was replaced with one consisting of the N-terminal 16 amino acids of the influenza haemagglutinin (HA) (HAQVP7, Fig. 1A), an integral membrane protein which is directed to the cell surface (Gething and Sambrook Nature 193: 620-625, 1981). However, this hybrid molecule was incorrectly processed by signal peptidase in vitro, being cleaved between gly 54 and ile 55 (Fig. 1B), even though the correct processing site for the HA signal peptide was conserved in the construction. The HA signal peptide was then fused to phe47 of VP7 to conserve the usual cleavage site of VP7 (HAFVP7 Fig. 1A). This precursor was correctly processed in vitro to yield a molecule indistinguishable from the wild-type (Fig. 1C and Stirzaker, et al. J. Gen. Virol. 67:245-2454, 1987). However, the fate of the protein in this case was remarkably different. While VP7 produced from the wild-type precursor remained intracellular (Fig. 2A), that derived from the hybrid



precursor was rapidly secreted from the cell and according to its increased size was modified with complex carbohydrate (Fig. 2B). These results therefore confirm that the H2 signal peptide has a dual function; it directs VP7 to the ER and has a role in retaining it there.

Inasmuch as the precursor HAFVP7 was correctly processed to yield VP7 which was secreted the present invention therefore provides a gene coding for a secreted VP7 protein with an N-terminus indistinguishable from that derived from the wild-type VP7 precursor protein. The only known modification to secreted VP7 is due to the addition of complex, endoglycosidase H-resistant carbohydrate attached at asn 69.

It is clear that the finding concerning the VP7 H2 signal peptide is applicable to other proteins naturally directed to the ER; their signal peptides may also play a similar role in ER retention. Fusion of these proteins to an appropriate foreign signal peptide derived from a protein naturally transported beyond the ER may also cause these proteins to be secreted.

The present invention consists in a gene including a sequence coding for a protein naturally retained in the endoplasmic reticulum and a sequence coding for a foreign signal peptide derived from a gene coding for a protein which is naturally transported beyond the endoplasmic reticulum, the gene sequence for the signal peptide being so fused to the gene sequence for the protein that cleavage of the protein from the signal peptide takes place at a site such that the antigenic characteristics and/or biological properties of the cleaved protein are the same as those of the naturally occurring protein.

In a further aspect the present invention consists in a vector, cell or organism carrying the gene according to the present invention. In a still further aspect the present invention consists in a process for the preparation of the protein comprising causing the gene to

be expressed in a culture medium and recovering the protein from the culture medium, and in the protein so expressed.

The protein may be any protein naturally retained in the ER including Rotavirus VP7 protein, glucose regulated proteins GRP78 (also known as BiP) and GRP94, protein disulphide isomerase, HMG CoA Reductase and adenovirus E19 protein. It is presently preferred, however, that the protein is Rotavirus VP7 protein. The term "protein naturally retained in the ER" is taken to mean proteins which are, in organisms in which they naturally occur, so retained. The term includes also derivatives of such proteins which correctly fold to the extent that they will still be transported out of the endoplasmic reticulum.

The signal peptide may be derived from any suitable protein that is naturally transported beyond the endoplasmic reticulum. These signal peptides include those from influenza haemagglutinin, from yeast invertase and from growth hormone, with the signal peptide from influenza haemagglutinin being preferred. Ideally, the nucleotide sequence of the gene should be such that the signal peptide is fused to the protein in a way which ensures that the cellular process of cleavage of the protein from the signal peptide occurs at the correct site on the protein i.e. at the point at which the protein would be cleaved from its natural signal peptide. If need be a suitable linker group may be included in the gene to ensure that the protein when processed has its natural N-terminal sequence.

The present inventors have made a further surprising discovery. They have found that proteins which are naturally resident in the cytoplasm, including the organelles present therein, may be correctly expressed and bound in the membrane of a cell if the gene for the protein is appropriately fused with a suitable signal peptide and a suitable transmembrane anchor domain.

In this aspect the present invention consists in a gene including:-

- (1) a sequence coding for a protein naturally retained in the cytoplasm of a cell;
- (2) a sequence coding for a signal peptide derived from a gene coding for protein which is naturally transported beyond the endoplasmic reticulum; and
- (3) a sequence coding for a transmembrane anchor domain, the three sequences being so fused that the gene will upon expression in a eukaryotic cell give rise to a correctly processed, appropriately folded, membrane bound version of the protein.

As would be understood by a person skilled in the art, the protein naturally retained in the cytoplasm may be from an organelle or a component of an organelle.

The anchor domain used in preferred embodiments of this aspect of the present invention may be derived from haemagglutinin, the VSV glycoprotein G, the IgG immunoglobulin protein, the histocompatibility antigen HLA-2A or from another C-terminally-anchored membrane protein. However, it is most preferred that the anchor domain is derived from haemagglutinin.

In an alternative preferred embodiment of this aspect of the present invention the signal peptide-anchor domain is derived from a type II membrane protein such as influenza neuraminidase, respiratory syncytial virus G protein or asialoglycoprotein, and most preferably influenza neuraminidase.

In a further aspect the present invention consists in a gene including:-

- (1) a sequence coding for a protein naturally exported from a cell; and
- (2) a sequence coding for a combined signal peptide-transmembrane anchor domain, the sequence coding for the signal peptide anchor domain being derived from a gene coding for a protein which is normally transported



beyond the endoplasmic reticulum,  
the sequence coding for the combined signal peptide-transmembrane anchor domain being fused in-frame to the sequence coding for the N-terminal region of the protein so that the gene will upon expression in a eukaryotic cell give rise to a correctly processed, appropriately folded, membrane bound version of the protein naturally exported from the cell.

The signal peptide-anchor domain is derived from a type II membrane protein such as influenza neuraminidase, respiratory syncytial virus G protein or asialoglycoprotein. At present it is preferred that the signal peptide-anchor domain is derived from influenza neuraminidase.

In other aspects the present invention consists in a vector, cell or organism carrying either of the genes defined above. In a still further aspect the present invention consists in a process for the preparation of the protein comprising the steps of causing the gene to be expressed and recovering the cells or the membrane therefrom to which the protein is bound. The invention still further consists in such a protein when bound to the surface of a eukaryotic cell. The invention still further consists in antigenic preparations containing such proteins and to antigenic preparations comprising suitable viral vectors containing these genes, with adenovirus and vaccinia virus being preferred vectors.

In a preferred embodiment the present invention further consists in a gene which, upon expression in a eukaryotic cell, gives rise to a correctly processed, membrane-bound version of VP7 which is no longer located on the ER but is transported to and displayed on the cell surface.

In order that the nature of the present invention may be more clearly understood, preferred embodiments thereof will now be described with reference to the following

examples and drawings, in which:-

Figure 1(A) shows the construction of VP7 genes with altered signal peptides. Bold type and lines indicate VP7 sequences: other sequences are derived from influenza haemagglutinin (CHO) Endo H-sensitive carbohydrate: (CHO\*\*\*) Endo H-resistant carbohydrate. Arrows indicate cleavage sites. (B and C) Partial N-terminal sequence of <sup>35</sup>S-methionine-labelled HAQVP7 and HAFVP7 after treatment of the protein with puromycin aminopeptidase (+). Radioactivity released after each cycle of Edman degradation was determined by liquid scintillation counting.

Figure 2 shows the cellular location and transport of VP7 produced from precursor H2VP7 (panel A) or HAFVP7 (panel B). Transfected COS cells were pulse-labelled for 15 min. with <sup>35</sup>S-methionine and chased for the times indicated. VP7 was recovered by immunoprecipitation.

Figure 3 shows the structure of HAFVP7A. Amino acid residues in italics are derived from haemagglutinin. Val 326 of VP7 is replaced by Ser and Ala is derived from the synthetic oligonucleotide. The arrow shows the cleavage site.

Figure 4 cellular location and transport of secreted variant HAFVP7 (tracks 1,2,5,6) or C-terminally anchored variant HAFVP7A (tracks 3,4,7,8). VP7 recovered from transfected COS cells by immunoprecipitation was digested with Endo H as indicated (+). Track 9 contains standard marker proteins of 30,46,60 and 92.5 kd.

Figure 5 sensitivity of proteins expressed in COS cells to digestion with endo glycosidase F. COS cells were transfected with HAFVP7 (tracks 1,2) or HAFVPYA (tracks 3,4) and intact cells were digested with endo F.

Figure 6 screening of vaccinia virus plaques with radiolabelled antibodies. Cells were infected with vaccinia virus strain WR or recombinants VV-VP7 or VV-HAFVP7A (Cell-surface variant). Plaques were incubated

with normal rabbit serum (NRS), mouse anti vaccinia (M- $\alpha$ -VV) or rabbit anti SA11 (R- $\alpha$ -SA11) serum incubated with iodine 125-labelled protein A.

#### Construction of novel VP7 genes

VP7 genes were constructed using procedures similar to those described in "Molecular Cloning: A Laboratory Manual", Maniatis et al. (1982) Cold Spring Harbor Press.

The SA11 VP7 gene was previously cloned into the XhoI site of the SV40-based expression vector pJC119 to create plasmid pHC9 (Poruchynsky, et al. J. Cell. Biol. 101:2199-2209, 1985). The gene encoding the VP7 precursor carrying the HA signal peptide i.e. HAFVP7 (Fig. 1A) was constructed in two stages as follows. Oligonucleotides of 65 and 58 bases encoding the first sixteen amino acids i.e. the signal peptide of the HA from the influenza strain A/NT/60/68/29C and the first 12 residues i.e. amino acids 51-61 of VP7 plus a linking ser residue were synthesized using an Applied Biosystems Model 380A DNA synthesizer. These oligonucleotides were complementary for 15 bases at their 3' ends. The synthetic oligonucleotides were phosphorylated, annealed and elongated using Klenow DNA polymerase to make them double stranded. The fragment was cut with XhoI and NcoI to generate 5' XhoI and 3' NcoI-compatible ends then ligated with a 4.3kb EcoRI-XhoI fragment and a 3.7kb EcoRI-NcoI fragment prepared from pJC9 to recreate the SV40 expression vector carrying the modified gene (HAQVP7) (Fig. 1A). The modified gene was also excised from this plasmid using XhoI and subcloned into the SalI site of the Bluescript KS M13+ vectpr (Stratagene) then transcribed into RNA in vitro. The RNA was translated in rabbit reticulocyte lysates in the presence of canine pancreatic microsomes under which conditions the processed VP7 protein was produced. N-terminal analysis of this species (Stirzaker & Both, (1989) Cell, In press) showed that processing had not occurred at the expected site i.e. at

glutamine 51, but four residues further downstream (Fig. 1B).

In view of this unexpected result the gene was further modified in an effort to obtain correct cleavage of the HA signal peptide from VP7. Single stranded template DNA was prepared for gene HAQVP7 using the helper phage M13K07 as described by Stratagene. An oligonucleotide was synthesized to insert 12 nucleotides coding for the amino acids phe-leu-arg-ala preceding the N-terminal glutamine residue of VP7. This mutation was constructed using the techniques and reagents provided in the Biorad mutagenesis kit, except that DNA was transformed into E.coli strain MV1190 and ampicillin resistant colonies were selected.

This Bluescript plasmid was digested with XhoI and NcoI to prepare the 5'-terminal fragment encoding the HA signal peptide now fused to the phe residue 47 of VP7. The HAFVP7 gene (Fig. 1A) in the SV40 vector was again constructed by three fragment ligation as described above. This gene could have been constructed in a single step if the signal peptide cleavage site could have been accurately predicted. The cleavage site of HAFVP7 translated in vitro was checked by N-terminal sequencing (Stirzaker et. al. 1989 Cell, In Press) and confirmed as correct (Fig. 1C).

The gene HAFVP7 was further modified to add a C-terminal transmembrane anchor domain as follows. The 3' terminal BamHI fragment of the VP7 gene in pJC9 was subcloned into M13mp10 and single stranded template DNA was prepared. An oligonucleotide complementary to bases 1014-1035 of the gene was synthesized with mismatched bases to introduce a BglI site at codons 325/326 of the VP7 gene. The oligonucleotide was elongated with Klenow DNA polymerase in the presence of DNA ligase to form double-stranded DNA which was transformed into E.coli strain JM101. Mutants were selected by hybridization with



the radiolabelled oligonucleotide at a temperature near its  $T_m$ .

A 528bp 5'HgaI-3' XhoI fragment was prepared from mutated, double-stranded M13 DNA and ligated with 4.83kb EcoRI-HgaI and a 4.3kb EcoRI-XhoI fragments from pJC9 to produce the gene VP7Bg1 in the SV40 vector.

The C-terminal membrane anchor domain of influenza haemagglutinin was prepared as follows. Oligonucleotides of 77 and 74 bases which were complementary for 13 residues at their 3' ends were synthesized, phosphorylated, annealed and elongated with Klenow DNA polymerase to make a 130bp double stranded fragment. This was digested with BamHI to produce termini compatible with the BglII site which had been introduced into VP7Bg1 as described above.

VP7Bg1 was cut with BglII (a unique site) and the membrane anchor fragment was incorporated into the plasmid by ligation. The plasmid was recut with BglII to eliminate molecules which had closed without acquiring the fragment and the DNA was transformed into E.coli RR1. Colonies carrying the transmembrane anchor fragment were identified by hybridization using one of the radiolabelled synthetic oligonucleotides as a probe. This plasmid was called VP7A.

The final construction of gene HAFVP7A in the SV40 vector was produced by three fragment ligation using the 5'XhoI-NcoI fragment from gene HAFVP7, the 4.3kb EcoRI-XhoI from pJC9 and the 3.8kb EcoRI-NcoI fragment from VP7A.

#### Expression of VP7 Genes in COS Cells

Wild-type and modified VP7 genes in the SV40 vector pJC119 were introduced into COS cells by electroporation (Chu et al. Nucleic acids Res. 15:1311-1326, 1987) using a Biorad Gene Pulser at a capacitance of 250uF with a pulse of 0.3Kv. Cells were allowed to recover at room temperature for 10-15 min. then gently resuspended in 3ml

of DMEM with 10% foetal calf serum and plated in a 60 mm dish. The following day the medium was changed to remove the dead cells. After 48 hrs  $^{35}\text{S}$ -methionine (150uCi/ml) was added to DMEM lacking methionine and serum and cells were labelled for 15 min. then chased for varying lengths of time with complete DMEM. Cells and medium were harvested, VP7 was recovered by immunoprecipitation and analysed by a gel electrophoresis and autoradiography (Poruchynsky et al., J. Cell Biol. 101:2199-2209, 1985). Digestion with Endo H and Endo F was carried out as previously described (Stirzaker & Both 1989 Cell, In Press).

The gene encoding the HAFVP7 precursor (Fig. 1) was fused with a C-terminal transmembrane anchor domain also derived from the influenza haemagglutinin gene. The DNA coding of this HA segment was spliced, inframe, to the VP7 gene at penultimate codon 325 and the stop codon of the HA segment was used to terminate protein synthesis (Fig. 3). This construction is called HAFVP7A.

When the genes encoding HAFVP7 and HAFVP7A were expressed in COS cells the fusion protein for the latter was slightly larger as expected (Fig. 4 compare tracks 5 and 7). The sensitivity of the carbohydrate attached to VP7 to digestion with Endoglycosidase H reflects the cellular location of the protein. Note that carbohydrate attached to HAFVP7 present inside the cells is completely Endo H sensitive reflecting its ER location (Fig. 4 track 6). HAFVP7 which has been secreted acquires Endo H-resistant carbohydrate during export (Fig. 4 tracks 1 and 2). In contrast, HAFVP7A is not secreted (Fig. 4 tracks 3 and 4) but has acquired some Endo H-resistant carbohydrate (Fig. 4 tracks 7 and 8) indicating that the intracellular proteins have left the ER and been transported, probably to the cell surface.

This was confirmed by incubating whole cells with endoglycosidase F, a protein which removes both simple and

complex carbohydrate from proteins. Intracellular VP7 produced in cells transfected with HAFVP7 was insensitive to endo F indicating that the cells remained intact during digestion (Fig. 5, tracks 1 & 2). In contrast, putative cell-surface expressed VP7 derived from HAFVP7A was sensitive to endo F (Fig. 5, tracks 3,4), indicating its accessibility and confirming its cell-surface location. It may also be possible to achieve cell-surface expression of other derivatives of VP7. For example, the present inventors have found that attaching the HA C-terminal anchor domain to the penultimate residue of the secreted variant deletion 47-61 (Poruchynsky et al, 1985) also results in surface presentation of the antigen. Similar results could be expected for related deletion mutations 43-61 and 42-61. However, for none of these variants is it known whether signal peptide processing occurs and the immunological effectiveness of these variant proteins is largely uncharacterized. It may also be possible to replace the HA segments with segments of equivalent function from other similarly transported proteins. However, in a number of different constructions that were tried, the hybrid VP7 protein was not transported to the ER. The reason for this is not clear but most likely relates to inappropriate folding of the protein caused by incorrect processing of the signal peptide or improper anchoring of the protein. Others have also noted that the simple addition of a membrane-spanning anchor domain to an otherwise secreted protein did not guarantee its successful transport to the cell surface (Langford et al Molec. and Cell. Biol. 6: 3191-3199, 1986).

It was previously demonstrated using recombinant vaccinia viruses that VP7 produced by expression of the wild-type gene was capable of inducing serotype-specific neutralizing antibodies in rabbits (Andrew et al. J. Virol. 61:1054-1060, 1987).

A recombinant vaccinia virus carrying the gene for

the cell-surface expressed VP7 was also constructed. Recombinant virus plaques expressing either cell-surface Vp7 or the wild-type protein were screened using a radiolabelled antibody. Plaques expressing the modified gene gave a strong signal compared with those carrying the wild-type gene which gave a weak signal (Fig. 6), further confirming the surface location of the modified antigen.

These recombinant viruses were used to vaccinate rabbits so that the antigenicity of the wild-type and modified proteins could be compared. Sera from the animals were assayed by a capture ELISA (Table 1). From the small number of animals tested so far the data indicate that the antigenicity of the cell-surface expressed antigen is considerably improved over the wild-type protein. These data are consistent with those of Langford et al (Molec. and Cell. Biol. 6:3191-3199, 1986) who observed a similar improvement in antigenicity by converting a soluble malaria antigen to a membrane-anchored form.

The conservation among VP7 proteins noted earlier (Gunn et al, J. Virol. 54:791-797, 1985; Gorziglia et al J. Gen. Virol. 67:2445-2454, 1986) will ensure that the principles established for the engineering and transport of the SA11 VP7 molecule will be applicable to the different rotavirus serotypes (defined by VP7) so that they can be similarly engineered to produce a multivalent vaccine.

By expression of the new gene using a vector such as adenovirus it will be possible to induce levels of antibodies which will actively protect against rotavirus infection. Alternatively, antibodies induced in colostrum and milk may be administered to offspring to provide passive protection against infection.



TABLE I. Immunization of rabbits with vaccinia virus recombinants carrying genes for wild type (wt) or cell-surface (sc) expressed VP7.

Rabbits*	Vaccinia virus	Day	Titre
1	VV-VP7 wt	0	< 33
		15	< 33
		21	< 33
		31	300
2	VV-VP7wt	0	< 33
		15	0
		21	0
		31	100
3	VV-VP7sc	0	100
		15	900
		21	8100
		31	2700
4	VV-VP7sc	0	< 33
		15	100
		21	300
		31	900

\*Rabbits were immunized at Day 0 and Day 21.

## CLAIMS:

1. A gene including a sequence coding for a protein naturally retained in the endoplasmic reticulum and a sequence coding for a foreign signal peptide derived from a gene coding for a protein which is naturally transported beyond the endoplasmic reticulum, the gene sequence for the signal peptide being so fused to the gene sequence for the protein that cleavage of the protein from the signal peptide takes place at a site such that the antigenic characteristics and/or biological properties of the cleaved protein are the same as those of the naturally occurring protein.
2. A gene as claimed in claim 1 in which the cleavage of the protein from the signal peptide takes place at the natural cleavage site.
3. A gene as claimed in claim 1 or claim 2 in which the protein is selected from the group consisting of rotavirus VP7 protein, glucose regulated proteins GRP 78 and GRP 94, protein disulphide isomerase, HMG CoA reductase and adenovirus E19 protein.
4. A gene as claimed in claim 3 in which the protein is rotavirus VP7 protein.
5. A gene as claimed in any one of claims 1 to 4 in which the signal peptide is derived from the gene coding for a protein selected from the group consisting of influenza haemagglutinin, yeast invertase and growth hormone.
6. A gene as claimed in claim 5 in which the signal peptide is derived from the gene coding for influenza haemagglutinin.
7. A gene as claimed in any one of claims 1 to 6 in which the protein is rotavirus VP7 protein and the signal peptide is derived from the gene coding for influenza haemagglutinin.

8. A vector containing a gene as claimed in any one of claims 1 to 7.
9. A cell containing a gene as claimed in any one of claims 1 to 7.
10. A process for the production of a protein naturally retained in the endoplasmic reticulum comprising culturing a cell as claimed in claim 9 in a culture medium and recovering the protein from the culture medium.
11. An antigenic preparation for use in raising antibodies active against a protein normally retained in the endoplasmic reticulum, the preparation comprising a protein produced by the process as claimed in claim 10.
12. An antigenic preparation as claimed in claim 11 in which the protein is rotavirus VP7 protein and the antigenic preparation is for use in raising antibodies active against rotavirus.
13. An antigenic preparation for use in raising antibodies active against a protein normally retained in the endoplasmic reticulum, the preparation comprising a suitable viral vector containing a gene as claimed in any one of claims 1 to 7.
14. An antigenic preparation as claimed in claim 13 in which the viral vector is either adenovirus or vaccinia virus.
15. An antigenic preparation as claimed in claim 13 or 14 in which the protein is rotavirus VP7 protein and the antigenic preparation is used to raise antibodies active against rotavirus.
16. A gene including:-
  - (1) A sequence coding for a protein naturally retained in the cytoplasm of a cell;
  - (2) A sequence coding for a signal peptide derived from a gene coding for a protein which is naturally transported beyond the endoplasmic reticulum; and
  - (3) a sequence coding for a transmembrane anchor domain,

the three sequences being so fused that the gene will upon expression in a eukaryotic cell give rise to a correctly processed, appropriately folded, membrane bound version of the protein naturally retained in the cytoplasm.

17. A gene as claimed in claim 16 in which the protein naturally retained in the cytoplasm of the cell is from an organelle or is a component of an organelle.

18. A gene as claimed in claim 16 in which the protein naturally retained in the cytoplasm of the cell is rotavirus VP7 protein.

19. A gene as claimed in any one of claims 16 to 18 in which the signal peptide is derived from the gene coding for a protein selected from the group consisting of influenza haemagglutinin, yeast invertase and growth hormone.

20. A gene as claimed in claim 19 in which the signal peptide is derived from the gene coding for influenza haemagglutinin.

21. A gene as claimed in any one of claims 16 to 20 in which the sequence coding for the transmembrane anchor domain is derived from a C-terminally-anchored membrane protein.

22. A gene as claimed in claim 21 in which the C-terminally-anchored membrane protein is selected from the group consisting of influenza haemagglutinin, VSV-G, IgG and the histocompatibility antigen HLA-2A.

23. A gene as claimed in claim 22 in which the C-terminally-anchored protein is influenza haemagglutinin.

24. A gene as claimed in any one of claims 16 to 18 in which the transmembrane anchor domain is derived from a type II membrane protein.

25. A gene as claimed in claim 24 in which the type II membrane protein is selected from the group consisting of influenza neuraminidase, transferrin receptor, respiratory syncytial virus G protein and asialoglycoprotein receptor.

26. A gene as claimed in claim 25 in which the sequence



coding for the transmembrane anchor domain is derived from the influenza neuraminidase.

27. A vector containing a gene as claimed in any one of claims 16 to 26.

28. A cell containing a gene as claimed in any one of claims 16 to 26.

29. A process for the production of a protein either naturally retained in the cytoplasm of a cell comprising culturing a cell as claimed in claim 28 in a culture medium and recovering the cells or the membrane therefrom to which the protein is bound.

30. An antigenic preparation for use in raising antibodies against a protein naturally retained in the cytoplasm of a cell, the preparation comprising a suitable viral vector containing a gene as claimed in any one of claims 16 to 26.

31. An antigenic preparation as claimed in claim 30 in which the viral vector is either adenovirus or vaccinia virus.

32. An antigenic preparation as claimed in claim 30 or 31 in which the protein is rotavirus VP7 protein and the antigenic preparation is used to raise antibodies active against rotavirus.

33. An antigenic preparation for use in raising antibodies active against a protein naturally retained in the cytoplasm of a cell, the preparation comprising the cells or membranes thereof produced by the process claimed in claim 29.

34. An antigenic preparation as claimed in claim 33 in which the protein is rotavirus VP7 protein and the antigenic preparation is used to raise antibodies active against rotavirus.

35. A gene including:-

- (1) a sequence coding for a protein naturally exported from a cell; and
- (2) a sequence coding for a combined signal

peptide-transmembrane anchor domain, the sequence coding for the signal peptide anchor domain being derived from a gene coding for a protein which is normally transported beyond the endoplasmic reticulum, the sequence coding for the combined signal peptide-transmembrane anchor domain being fused in-frame to the sequence coding for the N-terminal region of the protein so that the gene will upon expression in a eukaryotic cell give rise to a correctly processed, appropriately folded, membrane bound version of the protein naturally exported from the cell.

36. A gene as claimed in claim 35 in which the signal peptide-transmembrane anchor domain is derived from a type II membrane protein selected from the group consisting of influenza neuraminidase, transferrin receptor, respiratory syncytial virus G protein and asialoglycoprotein receptor.

37. A gene as claimed in claim 36 in which the type II membrane protein is influenza neuraminidase.

38. A vector containing a gene as claimed in any one of claims 35 to 37.

39. A cell containing a gene as claimed in any one of claims 35 to 37.

40. A process for the production of a protein naturally exported from a cell comprising culturing a cell as claimed in claim 39 in a culture medium and recovering the cells or the membrane therefrom to which the protein is bound.

41. An antigenic preparation for use in raising antibodies against a protein naturally retained in the cytoplasm of a cell or naturally exported from a cell, the preparation comprising a suitable viral vector containing a gene as claimed in any one of claims 35 to 37.

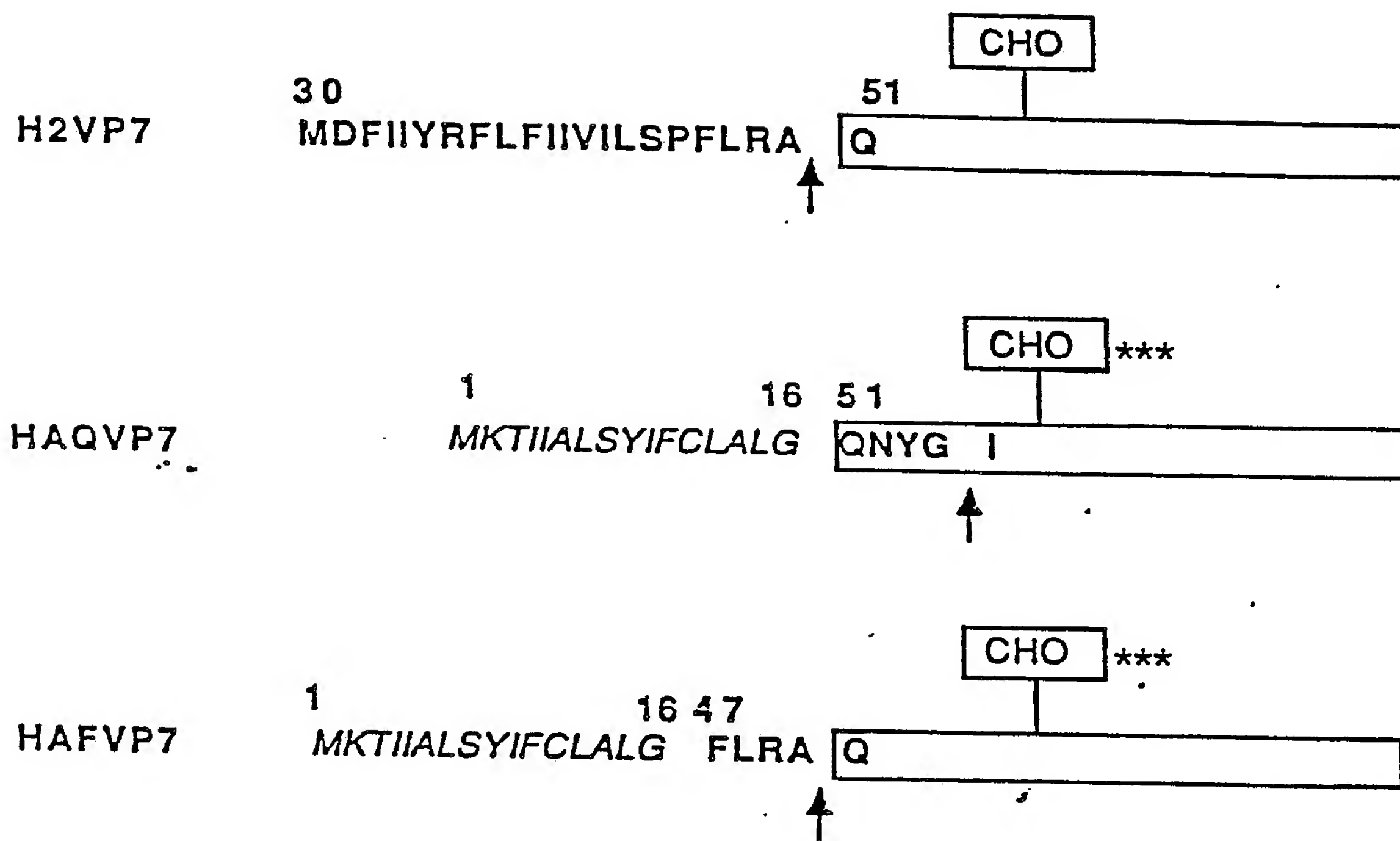
42. An antigenic preparation as claimed in claim 41 in which the viral vector is either adenovirus or vaccinia virus.

43. An antigenic preparation for use in raising antibodies active against a protein exported from a cell, the preparation comprising the cells or membranes thereof produced by the process claimed in claim 40.

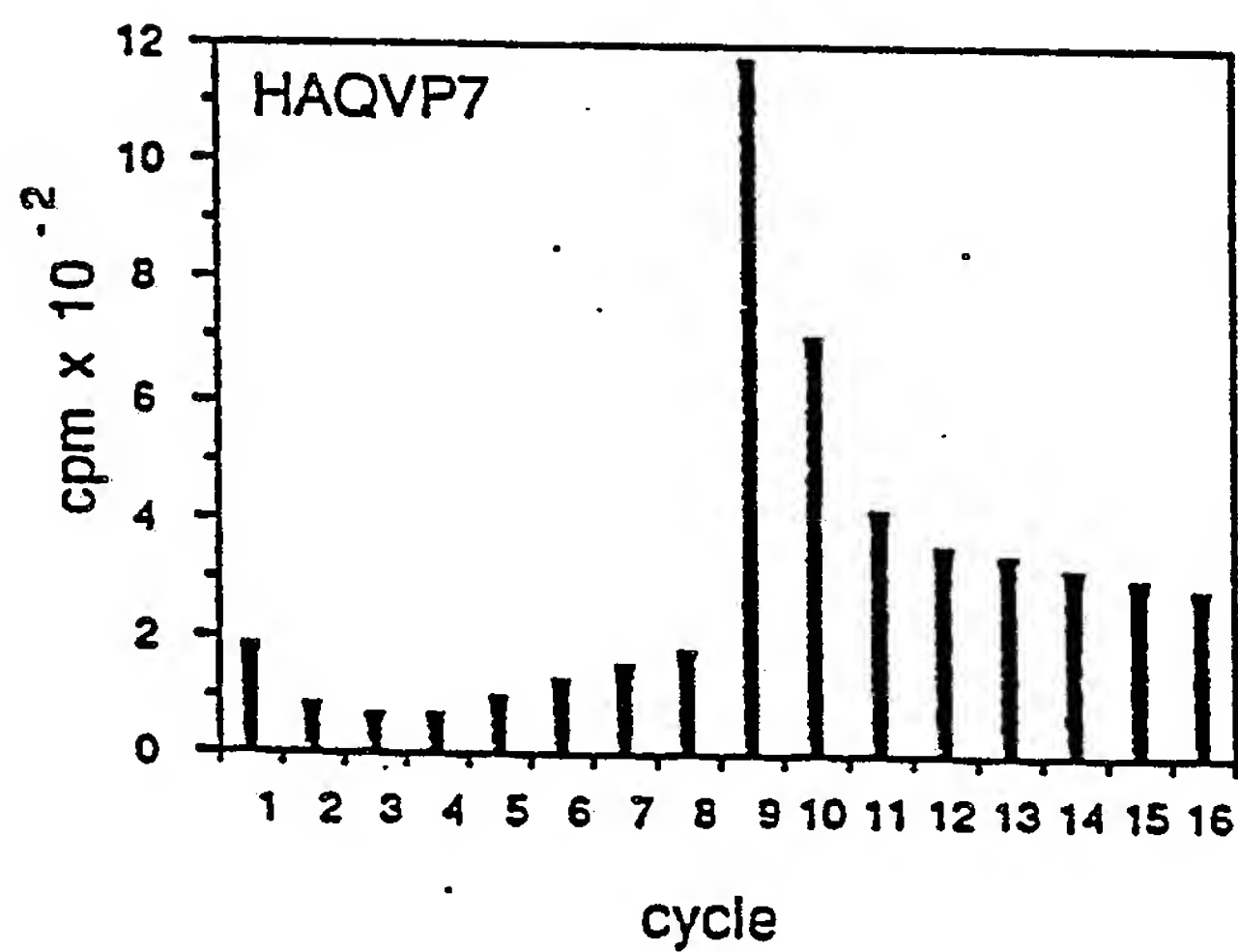
FIG 1

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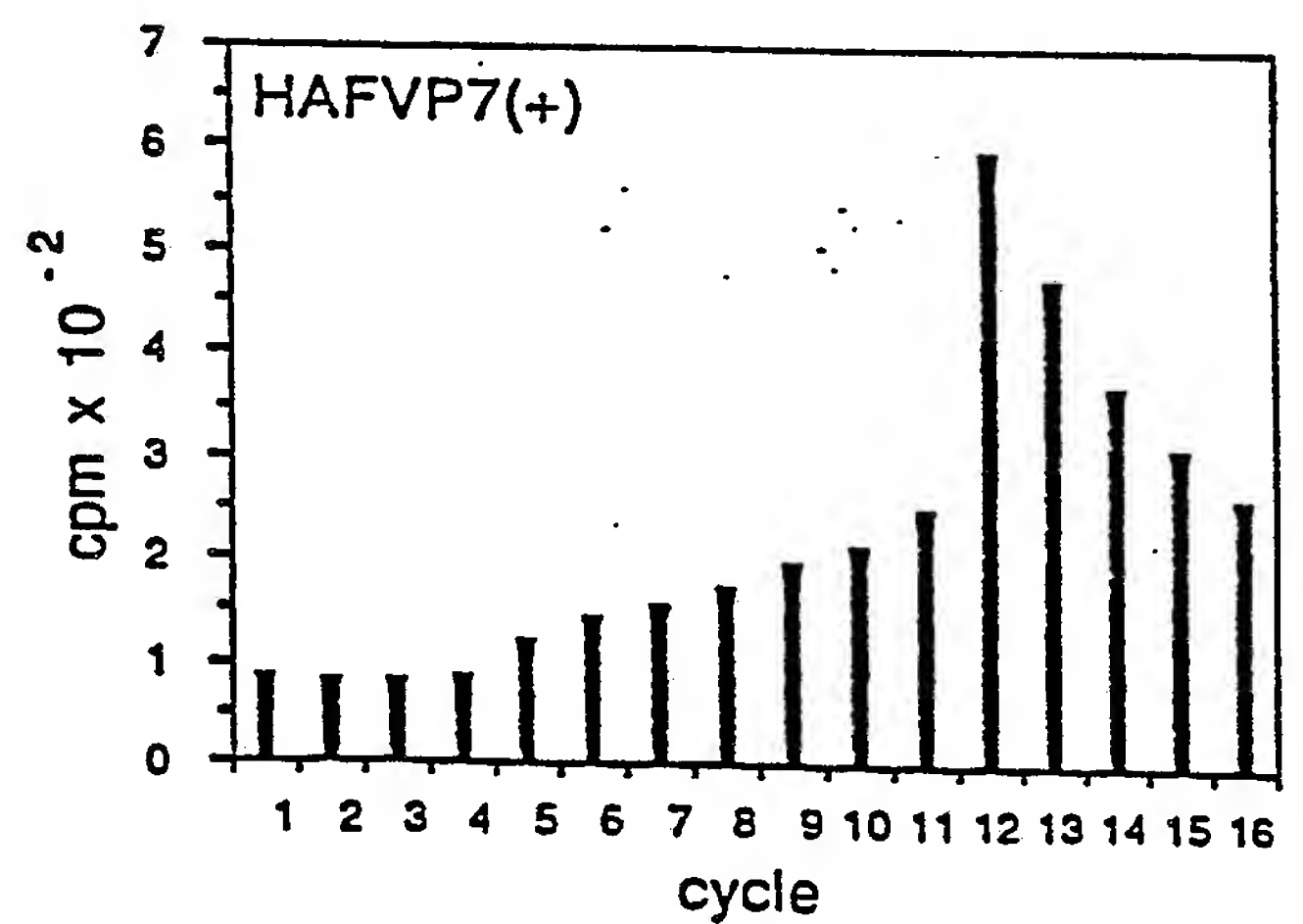
A



B



C

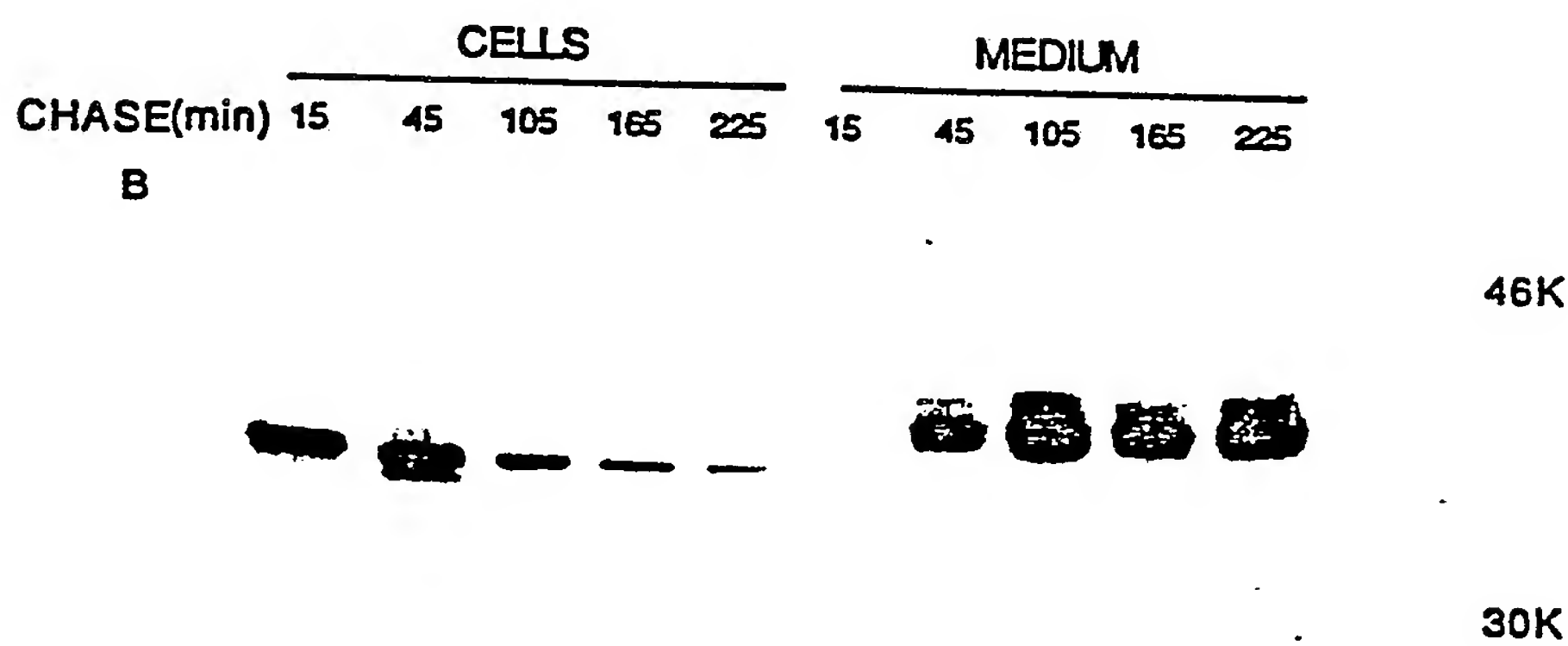
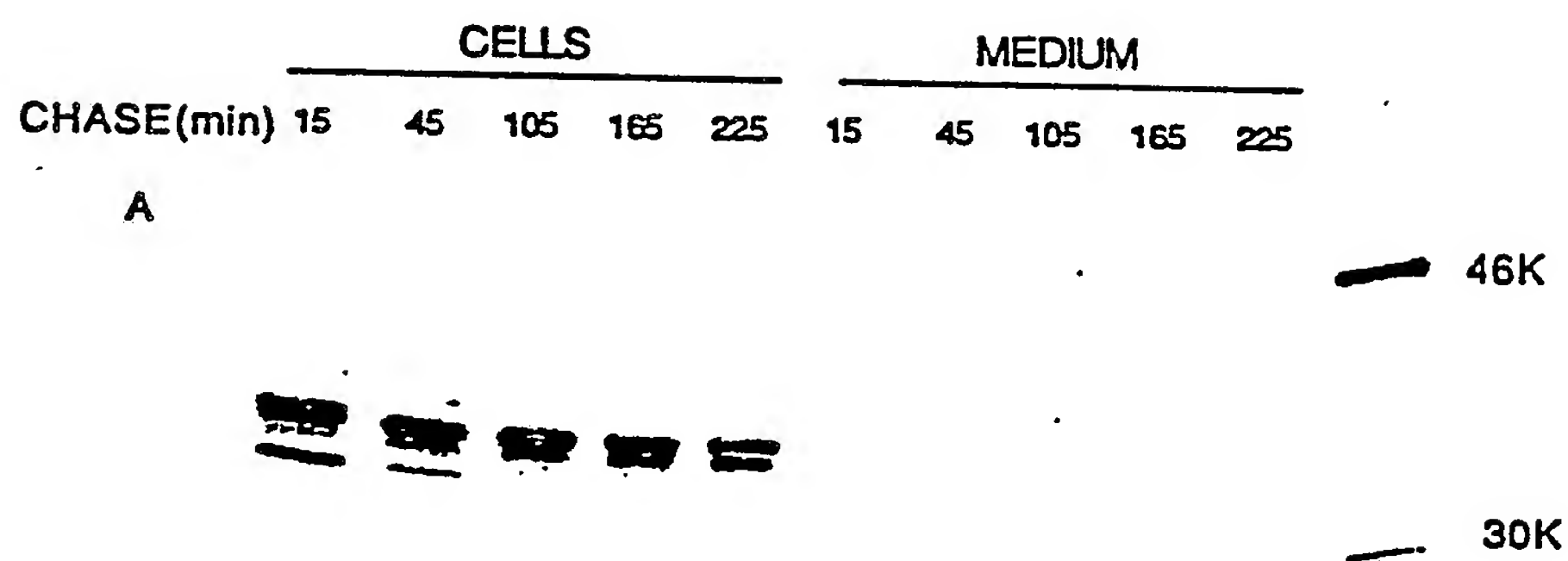


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FIG 2

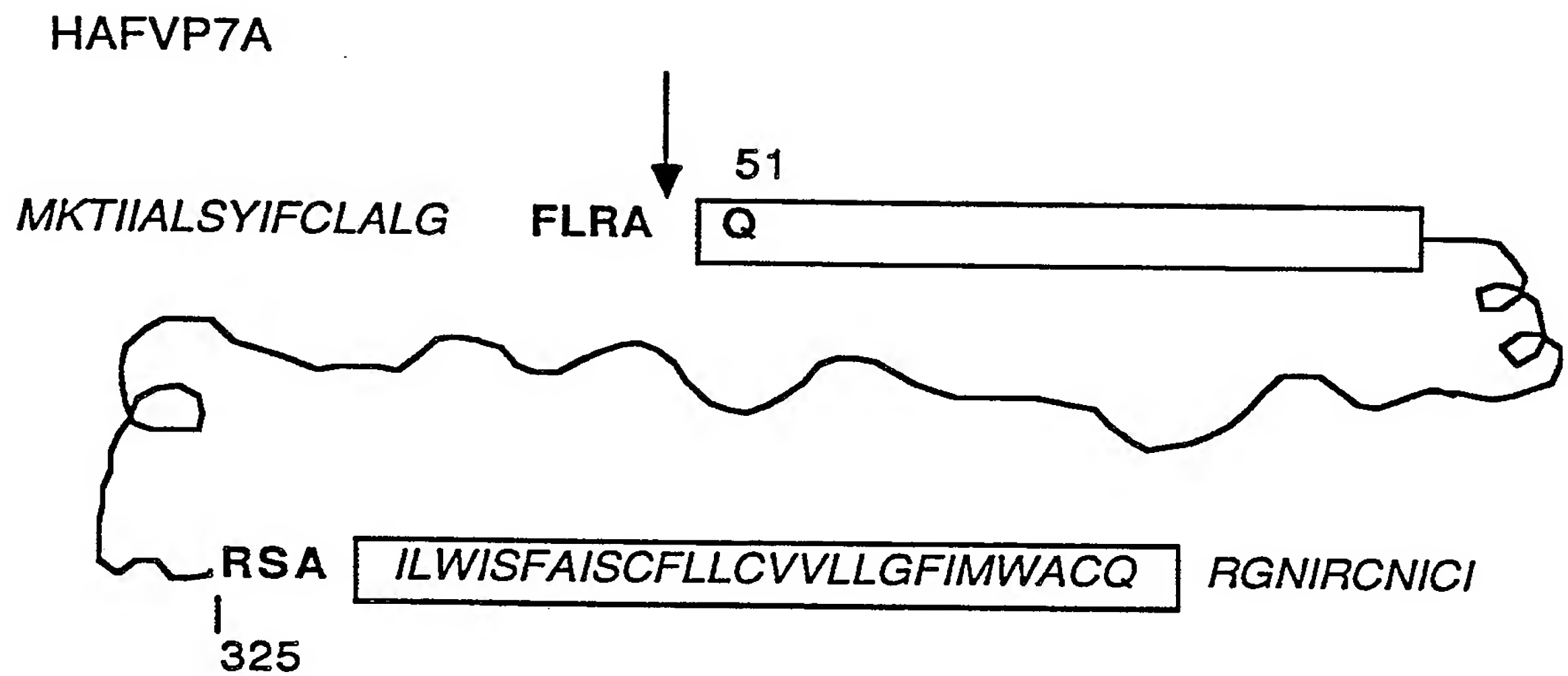
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Figure 3

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FIG 4

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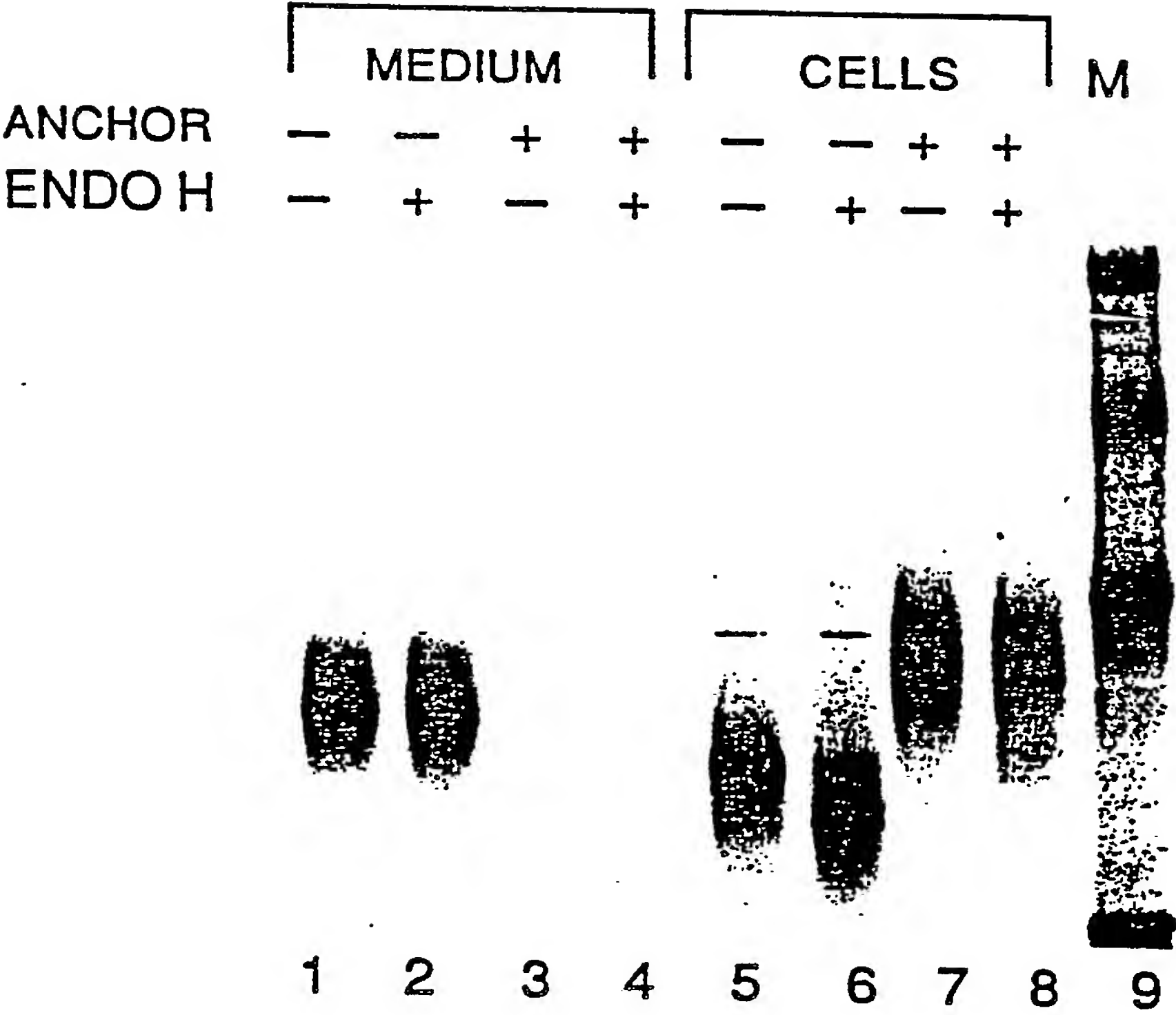
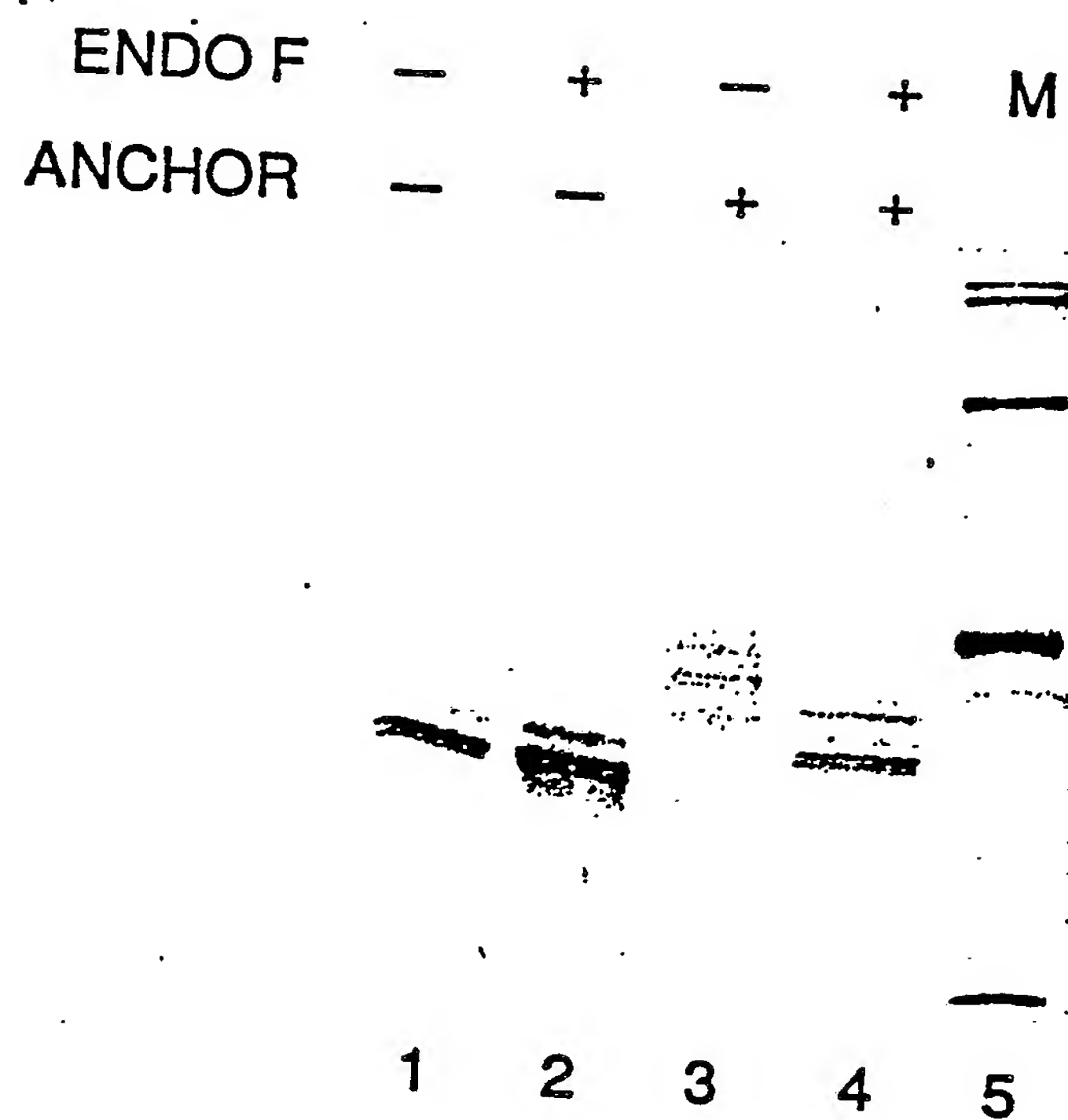


FIG 5

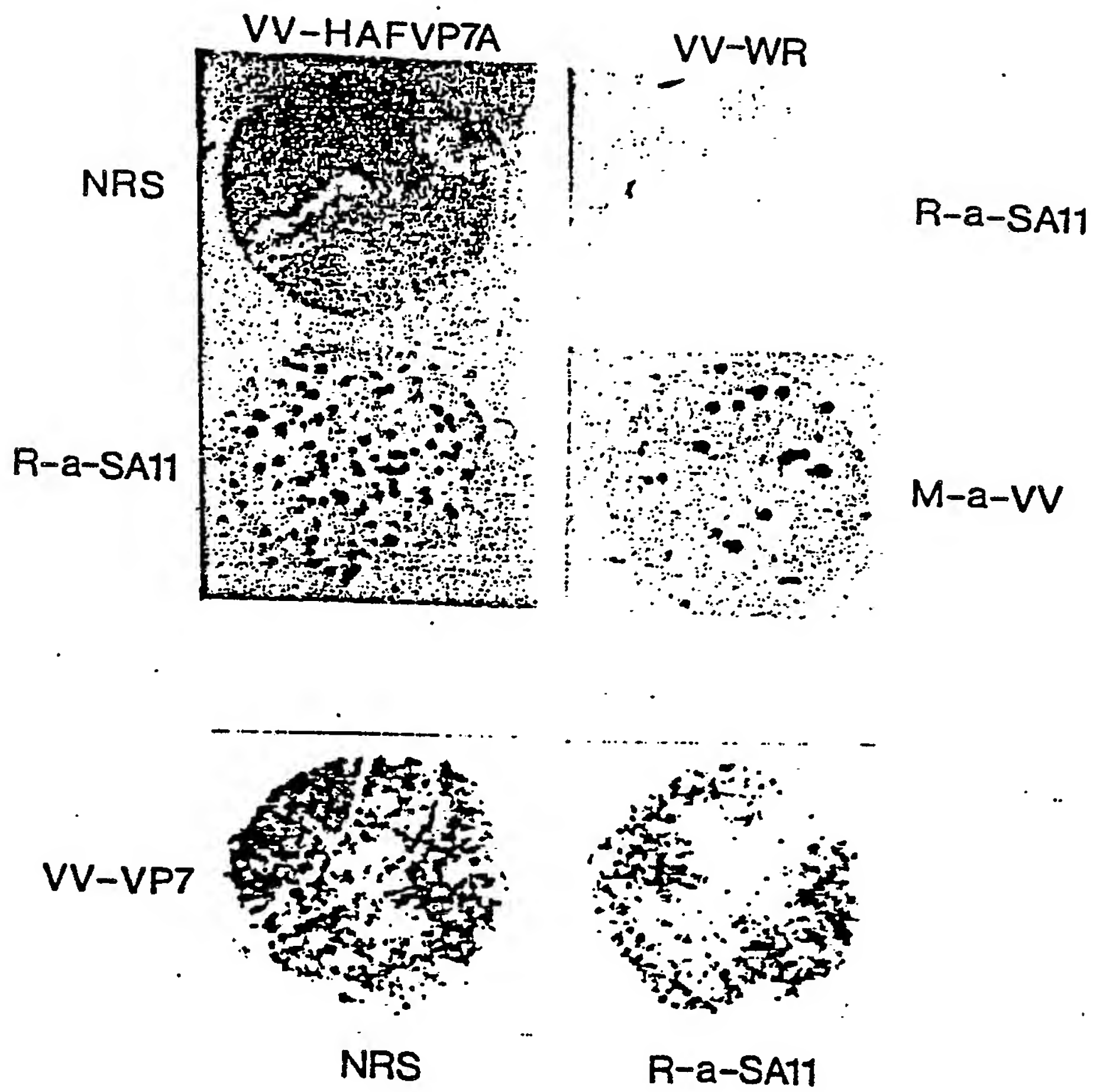
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FIG 6



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# INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 89/00038

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl. <sup>4</sup> C12N 15/00; C12P 21/02; C12N 5/00, 7/00; C07K 13/00; A61K 37/02, 31/73, 39/235, 39/285, 39/12		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC <sup>4</sup>	USPA, WPI, WPIL, USPA, Chemical Abstracts - Keywords as appended	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
AU : IPC C12N 15/00		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	J. Biol. Chem., Vol. 263 (32): 17084-17091 (15 November, 1988), R. Freudl et al "Dihydrofolate Reductase (Mouse) and beta-Galactosidase (E. coli) Can Be Translocated across the Plasma Membrane of E. coli"	(16,17,19-29)
X	J. Cell Biology, Vol. 107: 865-876 (September, 1988), S.W. Hiebert and R.A. Lamb "Cell Surface Expression of Glycosylated, Non-glycosylated, and Truncated Forms of a Cytoplasmic Protein Pyruvate Kinase"	(16,17,19-29)
X	Mol. and Cell. Biology, Vol. 8 (4): 1709-1714 (April, 1988), S. Vijaya et al "Transport to the Cell Surface of a Peptide Sequence Attached to the Truncated C Terminus of an N-Terminally Anchored Integral Membrane Protein"	(16,17,19-33, 35-43)
X	Science Vol. 238 (4831): 1280-3 (October, 1987), I.W. Caras et al "Signal for Attachment of a Phospholipid Membrane Anchor in Decay Accelerating Factor"	(35-40)
(continued)		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 15 May. 1989 (15.05.89)		Date of Mailing of this International Search Report 16 May 1989 (16.05.89)
International Searching Authority Australian Patent Office		Signature of Authorized Officer J. ASHMAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	J. Biol. Chem., Vol. 262 (17): 8416-8422 (15 June 1987), S. MacIntyre et al "The Signal Sequence of an E. coli Outer Membrane Protein Can Mediate Translocation of a Not Normally Secreted Protein across the Plasma Membrane"	(16,17,19-29)
X	Mol. and Cell. Biology, Vol. 6 (9): 3191-3199 (September, 1986), C.J. Langford et al "Anchoring a Secreted Plasmodium Antigen on the Surface of Recombinant Vaccinia Virus-Infected Cells Increases Its Immunogenicity"	(35-43)
D,X	J. Cell Biol., Vol. 101: 2199-2209 (December, 1985), M.S. Poruchynsky et al "Deletions into an NH <sub>2</sub> -Terminal Hydrophobic Domain result in Secretion of rotavirus VP 7, a Resident Endoplasmic Reticulum Membrane Glycoprotein"	(1-15)
X	AU,A, 73819/87 (ABBOT LABORATORIES, USA) 7 January 1988 (07.01.88)	(1-6,10-12)
X	WO, 06590/87 (BIOENTERPRISES PTY LTD, NSW, AUSTRALIA) 5 November 1987 (05.11.87)	(16-43)
X	AU,A, 63701/86 (THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH, VIC, AUSTRALIA) 12 March 1987 (12.03.87)	(16-43)
X	AU,A, 57362/86 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 20 November 1986 (20.11.86)	(1-15)
X	EP, 55942 (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK) 14 July 1982 (14.07.82)	(16,19-29, 35-40)

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

## II. FIELDS SEARCHED (contd)

Appended keywords.

Search 1 : signal and VP7 protein or GRP 78 or GRP 94 or protein disulphide isomerase or HMG Co A Reductase or E19 or glucose regulated protein.

Search 2 : (a) hybrid or fusion and membrane protein  
(b) anchor and signal or leader.

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 1, 2, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The scope of the terms "endoplasmic reticulum protein" and "foreign signal peptide" is indeterminate.

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

Claim 1 : gene sequence for endoplasmic reticulum protein linked to a foreign signal peptide.

Claim 16: gene sequence for a cytoplasmic protein linked to a transmembrane anchor and signal peptide.

Claim 35: gene sequence for an extracellular protein linked to a transmembrane anchor and signal peptide.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☒ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee. (claims 16 and 35)

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.